The University of Chicago

THE EFFECT OF VARIOUS STAGES OF VITAMIN A DEFICIENCY IN THE WHITE RAT ON RESISTANCE TO *NIPPOSTRONGYLUS MURIS*

A DISSERTATION SUBMITTED TO THE FACULTY OF THE DIVISION OF THE BIOLOGICAL SCIENCES IN CANDIDACY FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF BACTERIOLOGY AND PARASITOLOGY
1942

By
EDWIN G. RILEY

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THE EFFECT OF VARIOUS STAGES OF VITAMIN A DEFICIENCY IN THE WHITE RAT ON THE RESISTANCE TO *NIPPOSTRONGYLUS MURIS*

EDWIN G. RILEY

From the Department of Bacteriology and Parasitology, University of Chicago

For some years it has been accepted that vitamin A contributes in some way to the maintenance of bodily resistance to infection. The subject as related to bacterial infections is reviewed by Lassen[1] while Culbertson[2] discusses A-avitaminosis as it pertains to the parasitic infections. The literature, however, fails to reveal any reports of changes in resistance prior to frank avitaminosis. Robertson[3] stated, " . . . a considerable reduction of resistance to infections occurs in animals suffering from a marked and prolonged deficiency of the fat soluble vitamins." Concluding from the work of Zilva[4] Lassen[1] stated, " . . . it looks as if the actual decrease of resistance sets in very late in the course of avitaminosis (at any rate, this applies to A-avitaminosis)." McCoy[5] found that twice the number of *Trichinella spiralis* larvae occurred in rats infected after 2 weeks on a vitamin A deficient diet as in normal rats. This difference increased tenfold after 5 weeks upon the deficient diet. At neither time could vitamin A be found in the livers of the rats by chemical methods.

The work presented here attempts to answer the question of the time at which decreased resistance appears and the nature of the mechanism involved in this changed resistance.

MATERIAL AND METHODS

Parasite.—The life cycle of *Nippostrongylus muris* was first described by Yokogawa[6] who found that various larval stages develop on the ground from ova passed in the feces of an infected rat. The fully developed larvae are capable of passing through the unbroken skin of a rat when the opportunity is presented. The worms feed and grow while passing through the skin and are carried by the blood stream to the lungs. Here they grow further, break out into the alveoli and, by crawling up the bronchial tree, are finally swallowed. In the intestine the sexes become differentiated and copulation results in the production of ova to continue the life cycle.

N. muris lends itself to an experiment of this nature because a quantitative infection can be given, the course of the infection can be followed daily, and quantitative measures can be made of the degree of infection. Furthermore, considerable information is available from the extensive immunological studies made with this helminth by Taliaferro and Sarles.[7,8] It has previously been determined that there is a decreased resistance to *N. muris* in rats which are frankly deficient in vitamin A (Spindler[9]). In order to follow the vitaminotic state of the animals as

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1. Experimental Studies on the Course of Paratyphoid Infections in Avitaminotic Rats, Copenhagen, Levin and Munksgaard, 1931.
2. Immunity Against Animal Parasites. New York, Columbia University Press, 1941.
3. Medicine **13:** 123, 1934.
4. Biochem. J. **13:** 172, 1919.
5. Am. J. Hyg. **20:** 169, 1934.
6. Parasitology **14:** 127, 1922.
7. J. Infect. Dis. **59:** 207, 1936.
8. J. Infect. Dis. **64:** 157, 1939.
9. J. Parasitol. **22:** 72, 1933.

closely as possible, chemical assays of the vitamin A stored in the liver were made at various stages in the course of the vitamin A depletion. The chemical determinations supplemented the usual methods of following the degree of avitaminosis by weight changes and the appearance of symptoms of A-avitaminosis.

Animals.—Albino rats of mixed sexes with an average weight of 29 gm. (table 1) were obtained from a local breeder upon weaning. They were divided into 2 groups of the same average weight and immediately placed upon their respective diets.

TABLE 1.—*Average Weight of Rats on Vitamin A Deficient and Normal Diets*

Days After Placed on Diet	Deficient		Normal	
	No. of Rats	Average Weight (gm.)	No. of Rats	Average Weight (gm.)
0	76	29.6	42	29.9
5	70	31.5	41	40.4
14	61	47.6	35	63.3
21	53	55.7	29	87.1
28	44	58.3	23	109.3
35	37	61.3	17	126.5
42	13	59.4		
49	14	56.3		
56	14	55.8		
63	4 died			

Although most workers recommend a separate cage for each animal used in vitamin studies, the deficient rats in the present work were first kept 3 in a cage. As the experiment progressed and the total number of animals decreased, the number of rats in each cage was decreased. Preliminary work showed that the results obtained in this manner parallel those of other workers. Crowding had a slight effect upon the changes of weight in the deficient groups of rats. This effect is of interest only as it reveals the unreliability of variations in weight as a criterion of the degree of A-avitaminosis and will be discussed later. The normal control rats were kept in groups of 6 in larger cages. All animals were placed on wire screens.

Diets.—The vitamin A deficient diet consisted of:

Thin wheat starch	68%
Extracted casein	18%
Dried yeast	9%
McCollum's salt mixture No. 85	5%

This diet is also deficient in vitamin D, the lack of which is not attended by changes in resistance to bacterial infections (Lassen[1]) or *Ascaridia lineata* (Ackert and Spindler[10]), the only helminth infection in which the relationship has been tested.

The normal diet consisted of dog chow (Purina) since the difference between the increase in weight with it and with the deficient diet supplemented with cod liver oil was found to be unimportant for the purposes of this experiment.

It would be unsafe to say that all of the differences observed between the normal and experimental animals were due only to a lack of vitamin A. The normal diet may have contained some factors, as yet unknown, which enhanced resistance to this infection. In addition, starvation may have played a role, since after an extended period of time on the deficient diet, the food consumption of the rats was greatly reduced.

During the period when ova were produced, the infected normal rats were also placed upon the above deficient diet, except that the casein was not extracted. In addition, quantities of dried tomato pomace which had been extracted with boiling 95% alcohol 4 times, each time for 1 hour, were added up to 4% to the diet of both groups. This substance added bulk to the feces of the infected rats and was required especially by those rats in the later stages of the vitamin A depletion, since they ate little food. It was found that if the fecal output fell below 0.5 gm. per day, the determination of the number of ova contained in the sample was entirely unreliable. In all cases, food was supplied in excess.

Method of infecting.—The strain of *N. muris* and the technic of infecting

10. Am. J. Hyg. **9**: 292, 1929.

the rats were the same as those employed by Taliaferro and Sarles.[7,8]

Feces containing ova was mixed with granular animal charcoal and allowed to incubate at room temperature for 7–10 days. The infective larvae were then collected by means of the Baermann apparatus and washed several times in tap water. By means of dilution counts, the desired number of larvae for an infecting dose was finally concentrated in 0.1 cc. of water. The abdomen of the rat was shaved the day before infecting and was washed with soap and rinsed at the time of infecting. The animal was fastened with its abdomen up by means of a strap and was maintained in this position for 15 minutes after 0.1 cc. of the larval suspension was applied to the skin of the abdomen. In infecting each group, deficient and normal rats were alternated.

Five series (A, B, C, D, and E) each consisting of 6 normal and 6 deficient animals, were infected at various intervals (table 3 and fig. 1) after they were placed upon the diets.

Ova counting.—The number of ova passed in the feces each 24 hours was determined and the numbers produced each day during the course of the infection were used to indicate the degree of resistance to infection with the helminth.

The number of ova was obtained by weighing each 24 hour sample of feces; emulsifying a weighed portion, using from 10% to the total output depending upon the weight of the sample and the stage of the infection; and diluting with 100 cc. of water. From this dilution a 0.1 cc. amount was placed in a deep well slide, saturated salt solution was added to float the ova and a slide was placed over the surface. The number of ova was then counted under the low power of a microscope. Uusally, two 0.1 cc. samples, but more if the results varied greatly, were examined in this manner. By calculating the dilution-factor, the total number of ova passed in 24 hours was determined.

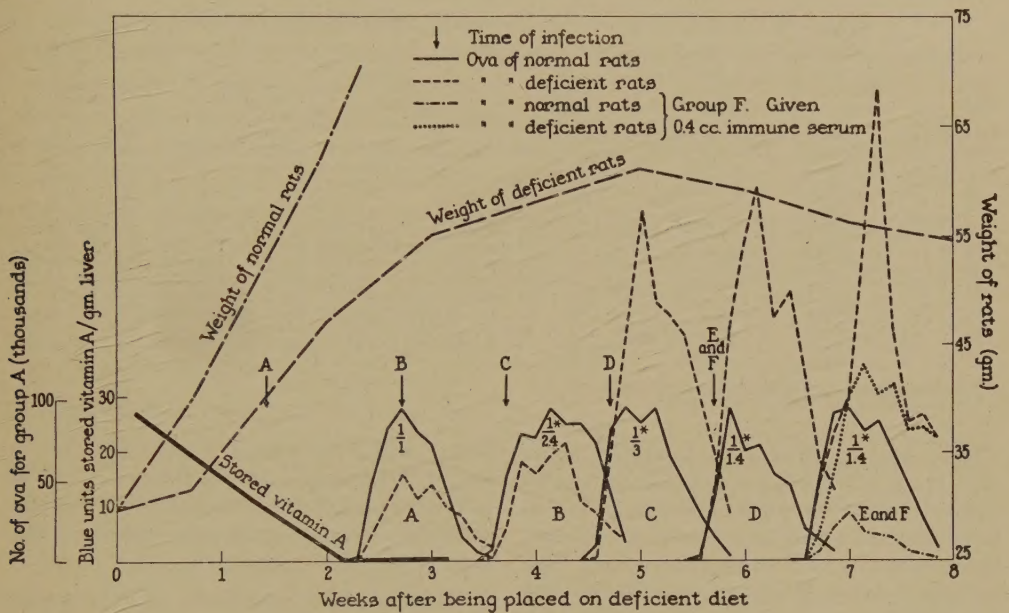

Fig. 1.—The vitamin A stored in the livers of the deficient rats, the weight changes, the time of infecting, and the daily average numbers of ova produced by the infected normal and deficient rats of groups A, B, C, D, E, and F (immune) as related to time after the rats were placed upon the deficient diet.

* Scale to which graph drawn as compared to that for group A.

Chemical determination of stored vitamin A.—The amount of vitamin A present in the livers of the rats was determined by means of the Carr-Price antimony trichloride reaction. The liver content of vitamin A was used as an indication of the vitaminotic state of the animal because this organ contains 90%

of the body store. It is generally believed that when vitamin A is no longer present in the liver, the other tissues are in a like state of depletion although recent work (Brenner[11]) would seem to indicate that some vitamin A may be present in cells of even frankly deficient animals.

TABLE 3.—*Daily Averages of Ova Output (in Thousands) for Each Group*

No. Days of Infection	A 10[a] 1300[b]		B 19[a] 2700[b]		C 26[a] 3100[b]		D 33[a] 2900[b]		E 40[a] 3000[b]		F 40[a] Immune 3000[b]	
	Deficient	Normal	Deficient	Normal	Deficient	Normal	Deficient	Normal	Deficient	Normal	Deficient	Normal
5	0	0	0	0	0	0	0	0	0	0	0	0
6	2±1	5±3	4±6	17±5	1±1	26±7	0	4±3	0	0	0	0
7	19±5	46±10	60±19	153±33	210±36	240±52	60±27	45±6	55±19	76±17	26±12	8±5
8	37±8	83±18	180±56	252±43	410±88	282±81	193±54	126±28	117±33	134±39	83±29	32±8
9	54±15	95±14	160±52	227±26	640±97	254±46	266±40	90±22	151±33	138±26	152±30	44± 9
10	40±8	77±16	192±54	290±43	475±106	281±45	282±105	96±32	271±61	118±41	185±18	26±8
11	47±14	70±15	216±43	260±40	452±127	189±19	210±86	70±24	433±68	129±32	155±29	22±8
12	22±10	40±11	108±36	264±68	416±88	140±44	228±100	62±28	211±51	96±24	163±27	21±10
13	29±12	15±4	88±19	213±69	300±89	83±37	151±32	21±16	127±24	66±22	120±39	8±6
14	14±5	10±5	59±26	113±22	195±72	40±33	93±34	17±19	132±29	37±23	123±34	4±4
15	9±5	5±6	33±20	30±10	85±21	5±5	62±18	6±7	113±22	10±10	113±34	2±2

[a] Number of days upon deficient diet at time infected.
[b] Number of larvae used to infect each rat.

A modification of the Davies[12] alkali-digest method was used in which each whole liver was removed from the rat, weighed, and dropped into boiling distilled water. After 5 minutes of boiling the liver was mashed with a glass spatula. Five per cent solution of potassium hydroxide was added, and the resulting mixture was boiled for half an hour while a small stream of nitrogen was constantly directed at the surface. At the end of this time, the mixture was transferred to a 100 cc. separatory funnel and allowed to cool. Redistilled ethyl ether was added to the funnel. The air above the mixture was washed out with a current of nitrogen and the funnel shaken thoroughly in the absence of light for 5 minutes. The water layer was removed and placed in another separatory funnel and the process repeated. The water fraction was then discarded and the 2 ether solutions added and washed with 5% solution of potassium hydroxide. This washing frequently had to be repeated in order to rid the ether layer of insoluble products of saponification. The ether solution was then washed 5 times with distilled water, the air being driven out of the funnel each time with nitrogen. The ether solution was then allowed to stand overnight in a dark place over anhydrous sodium sulphate. The next day the material was filtered through a sintered glass funnel and dried in a warm place with a stream of nitrogen passed over the surface of the solution. The residue was taken up in 2.0 cc. of redistilled chloroform, placed in a Wassermann tube and diluted in series by a factor of two in other (2–5) tubes (Wassermann) of uniform size, leaving 1.0 cc. in each tube. To each of these was added 1.0 cc. of a saturated chloroform solution of antimony trichloride and the resulting color compared to a standard color prepared after the method of Black and coworkers.[13] In some cases more chloroform was added in order to match the standard color. The blue color of the standard equaled that given by 6 blue units of vitamin A.

This method served to give a quantitative estimation of the vitamin present. Assays were carried out on 2 rats at various intervals (table 2 and fig. 1) after the animals had been placed upon the deficient diet until the presence of vitamin could no longer be determined by this method.

Passive immunization.—The immune serum was obtained from 16 rats which were each given 4 infecting doses of 3,000, 6,000, 10,000, and 15,000 larvae at weekly intervals. Five days after the last infection, the rats were bled with sterile precautions from the blood vessels of the axilla. The serum was separated and kept in a sterile vial at 4 C. until used 1 week later.

Each rat passively immunized was

11. Unpublished data.
12. Biochem J., **27**: 1770, 1938.
13. J. Am. Pharm. A. **27**: 199, 1938.

given 4.0 cc. of serum intraabdominally the day before being infected. Five normal and 5 deficient rats were used and comprised group F for which group E was used as a control.

N. Muris Infection at Various Stages of Vitamin A Deficiency

In following the symptomatic course of vitamin A deficiency in the rats, there was a normal increase in weight for 3 weeks (table 1 and fig. 1) after being placed on the deficient diet. The animals continued to gain weight from the third to the fifth week, although slowly. Rats of the size used typically show a cessation of the gain or a decline in weight after 3 weeks on the deficient diet. The continued gain in weight, in the present case, was due to the greater ease with which the deficient rats could feed as the number in each cage was reduced. A slow decline in weight started at the fifth week, and during the ninth week, 4 of the remaining 14 rats died of vitamin A deficiency. None of the animals developed xerophthalmia, although the characteristic findings of falling hair and general emaciation became evident after the third week of vitamin A depletion.

The increase in weight of the normal rats was rapid from the start, and, since their course was normal in every respect, was not followed after the fifth week.

At the beginning of the experiment, chemical assay of the stored vitamin A in the livers revealed an average of 27 blue units per gm. of liver tissue (table 2 and fig. 1). Eight days later this value became 11 units per gm. of liver tissue, and no more vitamin A could be found in their livers after the rats had been 15 and 22 days on the deficient diet. No further determinations were made.

The 6 normal and 6 deficient rats of group A were infected 10 days after being placed on their respective diets. Six days later, when ova were first found in the feces, the initial number (table 3 and fig. 1) from the deficient rats was less than that from the normal animals. The expected peak in the number of ova passed on the ninth day of infection and gradual fall in this number to near the base line by the 15th day were found in all cases. The deficient rats, however, exhibited a daily average at the peak of ova production about half that of the normal rats. The values for these 2 peaks were 54 ± 15 and 95 ± 14, respectively. The standard errors for these values were arrived at by using the formula $\frac{\text{S. D.}}{\sqrt{n-2}}$ in which S.D. is the standard deviation and n the number of rats in each group. The difference between the above mean values is but twice the standard error of the difference.

TABLE 2.—*Vitamin A Content of the Liver Determined Chemically*

Days After Placed on Diet	Weight of Liver (gm.)	Total Vitamin in Blue Units	Blue units of Vitamin A per gm. of Liver
1	2.07	48	23
	1.79	48	27
9	2.09	24	11
	2.12	24	11
15	3.54	0	0
	3.22	0	0
22	3.15	0	0
	3.61	0	0

After 19 days on their diets, the rats of group B were infected. They also showed ova in their feces for the first time on the sixth day, but the peak of the average daily number of ova from the deficient animals (216 ± 43) was slightly less than that from the normal ones (290 ± 43). The rats of groups C and D were infected 26 and 33 days, respectively, after being placed on the diets. The daily average numbers of ova from the deficient rats of both groups were approximately twice as high at the peaks

of ova excretion and were higher on the 15th day of infection than the numbers from the normal animals. The difference in the peaks was greater in group E (infected after 40 days on the diets) with a larger number of eggs passed on the 15th day of the infection from the deficient animals as compared to normal ones. The differences between the daily averages of ova production by the deficient and normal rats in groups C and D were 1.5 times and in group E was 4 times the standard errors of the differences. The results reported here, therefore, must be considered as an indication of a possible course of infection of rats with *N. muris* when the animals were at various stages of A-avitaminosis.

The peak in the daily average number of ova from the normal rats of group F which received immune serum was one-third that of the normal rats of group E. After the peak of ova excretion, the subsequent reduction in ova passed was more rapid in the immunized than non-immunized normal animals. The deficient rats of group F showed a peak in the daily average ova production which, although higher than that of the normal rats of group E, was about one-third as great as the peak in production of the deficient animals of group E. In this instance, however, the numbers of ova passed by the normal and avitaminotic rats on the 15th day of infection were the same.

Figure 1 shows the vitamin A contents of the livers, the changes in weights, the times of infecting the groups, and the daily average numbers of ova passed by the infected groups of rats as related to the time after the animals were placed on their diets. The changes in weight of the normal rats are not shown after the second week in order that those of the deficient animals may be better seen. The average numbers of ova passed daily are shown for group A. Since different numbers of larvae were used in infecting the different groups of animals, the curves for the numbers of ova passed by the normal rats in each group are all drawn to the same height at the peak of ova production to give a comparative representation. The scales to which they are drawn as compared to group A are indicated.

Time of Appearance of Decreased Resistance

The time of appearance of a decreased resistance to infection due to vitamin A deficiency must be considered in terms of the original amount of stored vitamin A and the amount supplied during the course of the depletion period. In the present case, the rats contained a small amount of stored vitamin which disappeared from the liver 2 weeks after the animals were placed upon the avitaminotic diet. A decrease in resistance to infection with *N. muris* did not appear until the rats had been on the deficient diet $4\frac{1}{2}$ weeks. After 2 weeks of vitamin A depletion, there was actually an increase in resistance to the helminth. This situation presented the unusual, but not unknown, condition in which subnormal animals manifest greater resistance than normal animals. Various other examples of this modified resistance have been reported: Spaeth and his colleagues[14] found that rats and guinea pigs which had been fatigued were more resistant to tetanus toxin than normal rested rats and guinea pigs. Rivers[15] states that some viruses are infective only for healthy animals. These instances and several others stand in opposition to the usual finding that any-

14. Am. J. Hyg. **2**: 51 and 527, 1922; **5**: 839, 1925.

15. Viruses and Virus Diseases, Stanford University, Calif., Stanford University Press, 1939.

thing which interferes with normal physiological mechanisms of animals renders them less resistant to infection.

Mechanism of Decreased Resistance

In endeavoring to determine the mechanism involved, we must ascertain the normal reaction of the host to this parasite as well as the changes which occur in the host as a result of the A-avitaminosis.

Due to the mode of entrance of *N. muris* into the body and its migration to the intestine, several tissue barriers must be traversed. The skin serves to prevent the passage of some of the worms. Work done by the author would indicate that an infection initiated by placing the larvae on the skin is less intense than one initiated with the same number of larvae injected subcutaneously. Taliaferro and Sarles[8] have shown that the worms which penetrate the skin cause a slight inflammatory reaction in the subcutaneous tissue which also retards some of the worms in their migration. Similarly, some worms are stopped in their passage through the lungs. After the parasites have reached the intestine and have become adults, an acquired immunity gradually manifests itself by causing degenerative changes in the worms starting about the tenth day and finally resulting in the elimination of most of the helminths around the 15th day. In an immune rat, the reaction of the host tissues at the barriers, mentioned above, is more intense with a more rapid mobilization of lymphocytic and phagocytic cells leading to a more effective blocking of the migration of the worms. The adult stage is also eliminated more rapidly from the intestine.

The pathology of A-avitaminosis has been the subject of numerous studies, most of which have been summarized in the recent report of Bessey and Wolbach.[16] From these studies, it appears that the skin is not less resistant to infection, although it undergoes various degenerative changes. The lungs exhibit a metaplasia of the respiratory epithelium to a stratified squamous type accompanied by hyperplasia. Vitamin A deficiency is associated with an increase in respiratory infections, usually a peribronchial pneumonia. Bessey and Wolbach[16] stated, however, that the changed type of epithelium of the lungs seems to protect the surrounding tissue from invasion with bacteria. The intestine, which is the final site of the parasite, reveals a metaplasia to the squamous type with hyperplasia. Verder,[17] Krakower and coworkers,[18] and Lassen[1] found that the intestinal wall is more permeable to the passage of bacteria. Krakower and coworkers,[18] in a recent study of schistosomiasis in vitamin A deficient rats, found that the cellular reaction in the liver which immobilizes and destroys the parasites is not as active as in normal rats. They found that although the lymphatic tissue of the body was reduced in extent, its activity was not impaired nor was that of the Kupffer cells of the liver. The ability of the vitamin A deficient animal to form antibodies seems little reduced as tested with various antigens (Werkman, Baldwin and Nelson,[19] Findlay and Maclean,[20] and Zilva.[4] Werkman[21] reported that the opsono-phagocytic index seemed little altered either in vitro or in vivo, whereas Gellhorn and Dunn[22] found

16. The Vitamins, Chicago, American Medical Association, 1939.
17. J. Infect. Dis. **4**: 589, 1928.
18. Puerto Rico J. Pub. Health & Trop. Med. **16**: 269, 1940.
19. J. Infect. Dis. **35**: 549, 1924.
20. Biochem. J. **19**: 63, 1925.
21. J. Infect. Dis. **32**: 255. 1923.
22. J. Nutrition **13**: 317. 1937.

that the index could be either increased or decreased with no apparent regularity.

It is not possible to supply an explanation for the increased resistance shown in the early stages of the vitamin A deficiency (group A).

No deviation from normal was observed in the rate of migration of the parasites through the avitaminotic rats in any of the groups as measured by the time ova were first found in the feces. Actually, ova were passed in larger numbers by the normal animals during the first days of the patent period. This difference, however, was due to the small numbers of ova and the differences in the amounts of feces excreted in the deficient and normal rats and did not occur when larger numbers of ova were produced. Groups C and D, in which a decrease in resistance was first observed, revealed a difference of about 1:2 between the daily average peaks of production of the normal rats and those deficient of vitamin A. The difference increased to 1:3 in group E. These findings would indicate a progressive decrease in resistance as the rats became more avitaminotic.

The deficient rats of groups C, D, and E exhibited considerable ability to rid themselves of the parasites as shown by the reduction to 13%, 22%, and 26%, respectively, in the numbers of ova passed on the 15th day of infection as compared to the peaks of ova excretion. On the same day of infection, however, the deficient animals of groups C, D, and E produced 17, 10, and 10 times, respectively, as many ova as compared to those passed by the normal rats in each of the groups. These facts suggest that after 4 weeks on the vitamin A deficient diet, the rats were less able to rid themselves of the infection than normal ones.

The passively immunized deficient and normal rats (group F) exhibited approximately one-third the daily average output of ova at the peaks of production as compared, respectively, to their controls, the deficient and normal rats of group E. Thus, it would seem that 4.0 cc. of immune serum were relatively as effective in reducing the degree of infection in each case. It must be noted, however, that the numbers of ova excreted by the immunized deficient rats (in group F) and the deficient controls (in group E) on the 15th day of infection were the same. This suggests that the immune serum delayed the migration of many of the parasites which finally established themselves in the small intestine.

Further evidence of the mechanism involved in the decreased resistance to *N. muris* infection associated with vitamin A deficiency was presented by Spindler.[9] Working with slightly immune (one previous infection with *N. muris*) rats, he found that many fewer worms were stopped in their passage through the lungs of deficient than of normal rats. This probably results from the less intense cellular reaction to an invading helminth in the tissues of A-avitaminotic rats observed by Krakower and others.[18]

Spindler[9] also found a larger number of adult worms in the small intestine of infected A-avitaminotic rats than in normal ones. From the data presented by him, it is found that more ova were produced per adult worm in deficient animals than in normal rats.

It may be said, then, that decreased resistance of vitamin A deficient rats to infection with *N. muris* manifests itself by an increase in the number of worms establishing themselves in the intestine, an increase in the number of ova produced per adult worm and an increase in the duration of infection. The first two factors combine to give an increased

output of ova. The changes mentioned above are probably in part due to a less intense cellular response of the host's tissues to the invasion of the parasites. After the peak of infection, the major part of the parasites are eliminated, showing the development of considerable immunity. On the other hand, the immunity which is developed is not as effective as that of the normal rat as shown by the increased duration of infection. Passive immunization of avitaminotic rats reduces the peak of infection relatively as much as in normal rats, although many of the parasites may be merely delayed in migration.

SUMMARY

A decrease in resistance to infection of A-avitaminotic white rats with *Nippostrongylus muris* found by Spindler is confirmed.

The decrease in resistance does not become evident until the rats have been upon the deficient diet for 4 weeks. After 2 weeks of vitamin A depletion, there is actually an increase in resistance to infection.

The changed resistance manifests itself by a more intense and prolonged infection.

Immune serum is relatively as effective in decreasing the peak of infection in both normal and deficient rats, although in the latter, many of the worms may merely be slowed in their migration through the body of the host.